Title Page (6-28-16, final clean version after accepted revisions)

Revival of Saprotrophic and Mycorrhizal Basidiomycete Cultures After Thirty Years in Cold Storage in Sterile Water

Dana L. Richter, Thomas G. Dixon and Jill K. Smith

School of Forest Resources and Environmental Sciences

Michigan Technological University

Houghton, Michigan 49931 U.S.A.

906-487-2149

dlrichte@mtu.edu

tgdixon@mtu.edu

jksmith@mtu.edu

Abstract

Vegetatively-colonized agar cores of 54 basidiomycete fungal isolates were stored at 5°C in tubes of sterile distilled water without manipulation for 30 years. The cultures represented 28 isolates of saprotrophic fungi and 26 isolates of mycorrhizal fungi. These cultures came from a group of 135 fungal isolates that were determined to be viable after 20 years of cold water storage. Overall, 47 of the 54 isolates (87%) grew vigorously when revived after storage for 30 years - of the 28 saprotrophic fungal isolates, 26 revived (93%); of the 26 mycorrhizal fungal isolates, 21 revived (81%). Eight of 13 isolates (62%) of *Laccaria* were viable after 30 years, which was considerably less viability than was found after 20 years for this genus of mycorrhizal fungi. However, a greater percentage of isolates of *L. bicolor* (83%) were viable than of *L. laccata* (43%) suggesting 30 years to be approaching the maximum limit for storage in cold sterile water for certain species. Considering the original 135 fungal isolates that were stored in sterile cold water from which this set was derived, overall survival after 30 years storage was 42%; however, saprotrophic fungi demonstrated considerably greater viability (70%) than mycorrhizal fungi (21%).

Key Words: culture maintenance, culture viability, fungal preservation, long-term storage, mycorrhizal fungi, saprotrophic fungi

Introduction

Long-term storage of fungus cultures to ensure isolate viability and stability is an important part of any mycology research laboratory. Interest in preserving microbial genomic diversity has also led to interest in long-term maintenance of cultures of fungi (Smith *et al.* 1994). However, genetic stability is not always assured after long periods of fungal growth. For example, characteristics of fungi such as pathogenicity, virulence, and growth rate are known to change over time when mycelium is continuously subcultured on agar (Marx and Daniel 1976, Hung and Molina 1986, Gramass 1991a, Gramass 1991b, Richter *et al.* 2004). Advantages and disadvantages of the various methods of maintaining fungus cultures over long periods of time for research are thoroughly discussed by Smith and Onions (1983). A simple method of maintaining fungus cultures that has been successful in several labs in recent years is storage under sterile water in refrigeration (Marx and Daniel 1976, Richter and Bruhn 1989a, Burdsall and Dorworth 1994, Richter 2008, Richter *et al*. 2010).

The simple storage of cultures in distilled water was introduced as early as 1939 by Castellani for human pathogenic fungi (Castellani 1939). The method became known by some as Castellani's method (Hartung de Capriles, *et al.* 1989). Hartung de Capriles *et al.* (1989) used Castellani's method to store 594 strains of fungi, composing 160 species of Ascomycetes (including Ascomycetous imperfect fungi) from a variety of substrates, including human pathogens, for from one to 20 years and found an overall survival rate of 62%. Ellis (1979) used Castellani's method to store 66 fungal isolates from a range of higher taxonomic groups and found only 52% overall viability after 20 months of storage, although basidiomycetes were particularly well

suited to this form of storage (73% survival). However, Castellani's method uses ambient temperature (approx. 20-25 ^{o}C) for storage of cultures under water, and researchers have since began storing cultures of fungi in water under refrigeration to increase survival (Marx and Daniel 1976, Richter and Bruhn 1989a, Burdsall and Dorworth 1994, Richter 2008, Smith *et al.* 1994, Richter *et al.* 2010).

Richter and Bruhn (1989a) stored 135 basidiomycete fungal isolates, represented by 83 species in 38 genera, in sterile cold water (5^{o}C); of those that were revived after eight to 48 months, 35 out of a total of 37 isolates of saprotrophic fungi were viable (95%), while only 53 out of a total of 98 isolates of mycorrhizal fungi were viable (54%). The original sterile water tubes containing the remaining cores of the isolates that were revived and viable were placed back in cold storage and left unmanipulated for 20 years. Due to difficulties inherent in storage over such an extended period, several tubes dried or became contaminated. Thus, after 20 years 69 isolates were revived (Richter 2008); 30 of 34 saprotrophic fungi were viable (88%), and 27 of 35 mycorrhizal fungi were viable (77%). Overall fungal viability of the 69 isolates after 20 years in cold storage in sterile water was 88% (Richter 2008).

The 57 fungal isolates with mycelial-agar cores remaining in the original tubes that were viable after 20 years storage in sterile water were placed back into the refrigerator and again left unmanipulated as in the previous studies. This paper reports the attempted revival of 54 of these basidiomycete fungal isolates after 30 years storage in cold sterile water (three isolates were lost due to drying or contamination).

Materials and methods

Fungal isolates were originally obtained from basidiome tissue. Saprotrophic fungi were isolated on potato dextrose agar (PDA, Difco) or 2% malt extract agar (2M, Difco), while mycorrhizal fungi were isolated on Modified Melin Norkrans agar (MMN, Marx 1969). Isolates were placed into sterile cold water storage 3 to 10 months after the time of isolation following the methods of Marx and Daniel (1976) (see Richter and Bruhn 1989a). In this process, a sterile cork borer (8 mm diameter) was used to cut colonized agar cores from the margin of actively growing cultures in petri dishes; agar was approximately 5 mm thick. Eight to 12 cores of each fungus were placed in 20 ml sterile distilled water in a 20 x 150 mm glass culture tube; screw tops were placed on tightly and sealed with several wraps of Parafilm® to minimize contamination or evaporation.

For revival, the last three to six cores of each isolate were taken out of the sterile water tube and placed mycelium-side down on the surface of fresh agar media in petri plates containing 2M for saprotrophic fungi or MMN for mycorrhizal fungi. Plates were incubated at room temperature and monitored biweekly for up to six weeks for evidence of growth. Growth was compared with an actively growing reference culture of the fungus species freshly transferred from an agar slant. After examination, isolates were rated either viable or non-viable based on resumed growth or lack of growth, respectively.

Results and Discussion

Results are shown in Table 1. Fungus species are grouped in families according to current taxonomy in *Index Fungorum* (Royal Botanical Gardens Kew, *et al.* 2016) as accessed March 2016. The 54 fungal isolates for which revival was attempted were represented by 37 species, in 23 genera, in 14 families; 28 of these isolates were saprotrophic fungi (24 species, in 16 genera and 8 families), and 26 isolates were mycorrhizal fungi (13 species, in 7 genera and 7 families). Of the 54 isolates, 47 (87%) grew vigorously when revived after 30 years storage in sterile cold water; 26 of the 28 saprotrophic fungal isolates revived (93%), and 21 of the 26 mycorrhizal fungal isolates revived (81%).

For most of the isolates that were viable, all of the cores that were retrieved from sterile water for each fungus grew new mycelium, except for *Amanita citrina, Suillus luteus,* and one isolate each of *Laccaria laccata* and *Tricholoma populinum*; for these isolates only one of the cores was viable (Table 1).

Species of *Laccaria* are important tree mycorrhizal fungi (for example, see Richter and Bruhn 1989b, 1993). Thirteen isolates of *Laccaria* in this study were represented by six isolates of *L. bicolor* and seven isolates of *L. laccata*. At 20 years (Richter 2008), all 13 of the isolates that were stored in cold sterile water revived when plated on agar medium. However, at 30 years, five of six isolates (83%) of *L. bicolor* revived, while only three of seven isolates (43%) of *L. laccata* revived. Though a small sample, this may indicate a difference in culture survivability between the two species. Other fungus genera that form mycorrhizae with trees fared well after 30 years

of storage, for example, three species of *Amanita* and four isolates representing two species of *Suillus* were viable.

A large family of principally saprotrophic fungi, the Tricholomataceae (*sensu lato*), were generally viable, with 11 of 11 isolates reviving after 30 years; two of these isolates are mycorrhizal fungi (*Tricholoma populinum* and *T. resplendens*). Good viability was also exhibited in the saprotrophic families Agaricaceae (all four isolates viable), Strophariaceae (all three isolates viable), and Pleurotaceae (all three isolates viable).

The fungi in this study were essentially a select group of isolates, in that these were isolates that had been stored, revived and survived up to 20 years in cold water storage from the previous study (Richter 2008). When percentage survival is calculated based on the original number of isolates stored (135 minus 19 isolates not attempted after 20 years, and 3 isolates not attempted currently due to drying or contamination), this results in an overall survival rate after 30 years of 42% for all fungi. However, this percentage is lowered by the high number of mycorrhizal fungi that did not survive approximately two to four years in the original set (Richter and Bruhn 1989). For the saprotrophic fungi alone, the survival rate from the original isolates after 30 years storage was 70%. In contrast, for the mycorrhizal fungi the survival rate after 30 years was only 21%. The percentage survival for the mycorrhizal fungi is skewed downward slightly due to the large number of isolates of *Scleroderma* (22) in the original set that had very low survival even after just 12 months of storage (Richter and Bruhn 1989a). If isolates of *Scleroderma* are removed from the original data set, percentage survival of mycorrhizal fungi after 30 years in sterile cold water becomes 28%, which is still considerably lower than saprotrophic fungi.

Table 2 compares survival by genus after approximately two to four years (Richter and Bruhn 1989a), 20 years (Richter 2008), and 30 years of storage (this study) for those genera where two or more isolates were originally stored in sterile cold water. Genera of both mycorrhizal and saprotrophic fungi vary in their survivability after long-term storage in sterile cold water. For example, as mentioned above, the mycorrhizal genus *Laccaria* (particularly *L. bicolor*) appears well-suited for sterile cold-water storage. In contrast, the mycorrhizal genera *Boletus, Lactarius, Paxillus, Scleroderma* and *Thelephora* appear unsuited for this type of culture storage. Other mycorrhizal genera were intermediate in their survival success among isolates after long-term storage. For saprotrophic fungi, three of three isolates of *Armillaria,* three of three isolates of *Pleurotus,* and two of two isolates each of *Hygrophoropsis, Lepista* and *Lycoperdon* survived after 30 years of storage. Other saprotrophic genera were variable in their survival success after 30 years.

Burdsall and Dorworth (1994) also demonstrated a high rate of survivability (94%) of saprotrophic basidiomycete fungus cultures in sterile cold water (5 ^{o}C) for up to seven years of storage. However, Johnson and Martin (1992), who stored cultures of saprotrophic basidiomycete fungi at 20 ^{o}C in sterile water for ten years reported only 26% survival; this suggests temperature to be a critical factor in the survival of cultures over extended periods. In contrast to saprotrophic fungi, Smith *et al*. (1994) stored 169 cultures of mycorrhizal fungi for up to 20 months in sterile water at 18 ^{o}C and showed that 84%-89% of cultures revived, while at 4^{o}C only 78% of cultures revived. Though a relatively short storage period, species differences among studies may account for slight differences seen in overall survival rate between storage

temperatures. Marx and Daniel (1976), who stored mycorrhizal fungus cultures for up to three years in sterile cold water (5 ^{o}C), showed that survival went down from 100% to 95% to 64% after one, two and three years, respectively.

It is of further interest that 15 of the isolates that were revived after 30 years in sterile cold water in this study did not survive after eight to 12 years of transfer annually on agar slants (see Table 1). This was true for seven of the 13 *Laccaria* isolates, indicating sterile cold water to be a superior method for maintaining isolates of this genus. Three of four isolates of the mycorrhizal genus *Suillus* also fared better stored in sterile cold water than by transferring annually on agar slants.

Sterile cold water storage is a simple and effective method of long-term storage of basidiomycete fungus cultures, however, functional group and family must be taken into account when considering this method for use by laboratories. Based on this study, this method of long-term storage is suitable for many isolates of saprotrophic basidiomycete fungi, but the same generalization cannot be made for mycorrhizal fungi. Families and genera of mycorrhizal fungi exhibit a variable response to long-term storage in sterile cold water. Although sterile cold water storage appears to be a good method to store isolates of the mycorrhizal genus *Laccaria* for at least up to 20 years, especially *L. bicolor*, the method does not appear suitable for storing isolates of many other genera.

While viability of fungal isolates is essential for any method of culture storage, effectiveness in service or maintenance of virulence of isolates is the assurance of success of a storage method.

Richter *et al.* (2010) compared 14 strains of wood decay fungi stored for 18 years on agar and transferred annually or stored in sterile cold water unmanipulated for the same amount of time, and found no significant difference ($p \leq 0.05$) in the amount of decay due to storage method for 12 of the isolates. For the two isolates that differed in decay rate, one isolate stored on agar resulted in greater decay, and one isolate stored in water resulted in greater decay. Four isolates of mycorrhizal fungi that revived after 30 years in the present study (*H. crustuliniforme* DR-32, *L. bicolor* DR-91, *L. laccata*, DR-137, *S. neoalbidipes* DR-44), were taken out of cold water storage after 24 years and inoculated onto red pine (*Pinus resinosa* Ait.) seedlings using methods of Richter and Bruhn (1989b). The four isolates were successful at forming a mycorrhizal mantle on root tips, confirming that the fungi were still effective at forming mycorrhizae (J.K.S. and D.L.R., unpublished data). Results of these decay and mycorrhizae tests suggest that the 54 fungal isolates of saprotrophic and mycorrhizal fungi in this study that remained viable after 30 years in sterile cold water would retain their virulence and be effective in the ecological role from which they were originally obtained.

Acknowledgements

The former Institute of Wood Research and the School of Forest Resources and Environmental Science, Michigan Technological University, provided facilities for long-term maintenance of these fungus cultures. Part of this work was conducted through a Summer Undergraduate Research Fellowship grant from Michigan Technological University. We wish to thank two anonymous reviewers for their helpful comments that improved the paper.

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Table 1. Survival of 54 basidiomycete cultures in sterile cold water (5°C) after 30 years of storage.

Fungus species by order and family	Isolate No.*	Mycorrhizal (M) or Saprotrophic (S)	Viability† +/-
AGARICALES			
Agaricaceae			
Calvatia gigantia	DR-105	S	+
Leucoagaricus naucinus	DR-83	S	+
Lycoperdon muscorum	DR-98	S	+
Lycoperdon perlatum	DR-84	S	+
Amanitaceae			
Amanita citrina (Schaeff.)	DR-35	M	+1
Amanita flavoconia	DR-94	M	+
Amanita muscaria	DR-59	M	(+)
Cortinariaceae			
Hebeloma crustuliniforme	DR-32	M	+
Hebeloma sp. "A"	DR-11	M	+
Hydnangiaceae			
Laccaria bicolor	DR-64	M	(+)
Laccaria bicolor	DR-72	M	(+)
Laccaria bicolor	DR-91	M	+
Laccaria bicolor	DR-100	M	(-)
Laccaria bicolor	DR-112	M	(+)
Laccaria bicolor	DR-141	M	(+)
Laccaria laccata	DR-5	M	(-)
Laccaria laccata	DR-95	M	(+)
Laccaria laccata	DR-102	M	(+)
Laccaria laccata	DR-113	M	(-)
Laccaria laccata	DR-115	M	(+1)
Laccaria laccata	DR-133	M	(-)
Laccaria laccata	DR-137	M	-
Lyophyllaceae			
Hypsizygus tessulatus	DR-129	S	(+)
Lyophyllum decastes	DR-87	S	(-)
Mycenaceae			
Panellus stypticus	DR-106	S	+

Physalacriaceae			
Armillaria mellea sensu lato	DR-52	S	+
Armillaria mellea sensu lato	DR-86	S	+
Armillaria gallica	DR-140	S	+
Hymenopellis colensoi	DR-88	S	-
Pleurotaceae			
Pleurotus ostreatus	DR-85	S	+
Pleurotus ostreatus	DR-93	S	+
Pleurotus ostreatus	DR-153	S	+
Strophariaceae			
Hypholoma sp.	DR-145	S	+
Pholiota sp. "A"	DR-50	S	+
Pholiota sp. "B"	DR-146	S	+
Tricholomataceae *(sensu lato)*			
Ampuloclitocybe clavipes	DR-38	S	(+)
Cantharellula umbonata	ATCC 62011	S	+
Clitocybe rivulosa	DR-33	S	+
Clitocybe gibba	DR-16	S	(+)
Clitocybe phaeophthalma	DR-67	S	+
Clitocybe sp.	DR-7	S	+
Collybia sp.	DR-40	S	+
Lepista glaucocana	DR-138	S	+
Lepista nuda	DR-147	S	+
Tricholoma populinum	DR-149	M	+1
Tricholoma sulphuresens	DR-79	M	(+)
BOLETALES			
Hygrophoropsidaceae			
Hygrophoropsis aurantiaca	ATCC 60968	S	+
Hygrophoropsis aurantiaca	DR-66	S	+
Rhizopogonaceae			
Rhizopogon ochraceorubescens	DR-128	M	+
Suillaceae			
Suillus luteus	DR-37	M	(+)

Suillus luteus	DR-82	M	(+1)
Suillus neoalbidipes	DR-9	M	(+)
Suillus neoalbidipes	DR-44	M	+
RUSSULALES			
Russulaceae			
Lactarius rufus	DR-71	M	+

† Three to six agar cores were plated for each fungus. +, indicates 3 or more cores were viable; +1, indicates only one of the cores was viable; -, indicates none of the cores were viable. Data in parenthesis indicate that the culture did not survive after 8-12 years on agar slants transferred annually.

Table 2. Summary of survival by genus of basidiomycete cultures in sterile cold water (5oC) after 2 to 4, 20 and 30 years of storage (only genera with two or more original isolates stored are shown).

Fungus Genus	Mycorrhizal or Saprotrophic	Total Number of Isolates	Number of Isolates Surviving After 2– 4 yrs*	Number of Isolates Surviving After 20 yrs †	Number of Isolates Surviving After 30 yrs
Amanita	M	9	3	3	3
Armillaria	S	3	3	3	3
Boletus	M	9	1	1‡	--
Clitocybe §	S	8	7	5	5
Hebeloma	M	4	3	2	2
Hygrophoropsis	S	2	2	2	2
Laccaria	M	15	13	13	8
Lactarius	M	9	1	1	1
Leccinum	M	2	1	0	--
Lepista	S	2	2	2	2
Lycoperdon	S	2	2	2	2
Paxillus	M	3	1	0	--
Pholiota	S	3	3	2	2
Pleurotus	S	3	3	3	3
Scleroderma	M	22	1	0	--
Suillus	M	13	7	4	4
Thelephora	M	3	0	--	--
Tricholoma	M	4	3	2	2

* Data from Richter and Bruhn 1989.

† Data from Richter 2008.

‡ The isolate of *Boletus* was lost between 20-30 yrs.

§ Includes *Ampuloclitocybe clavipes*